

The SH2 domain protein GRB-7 is co-amplified, overexpressed and in a tight complex with HER2 in breast cancer

EXHIBIT B

D.Stein, J.Wu, S.A.W.Fuqua¹ C.Roonprapunt, V.Yajnik, P.D'Eustachio², J.J.Moskow³, A.M.Buchberg³, C.K.Osborne¹ and B.Margolis⁴

New York University Medical Center, Department of Pharmacology, Kaplan Cancer Center and ²Department of Biochemistry, 550 First Avenue, New York, NY 10016, ³Department of Microbiology and Immunology, Jefferson Cancer Institute, Thomas Jefferson Medical College, Philadelphia, PA 19107 and ¹Division of Medical Oncology, Department of Medicine, University of Texas Health Sciences Center at San Antonio, San Antonio, TX 78284, USA

⁴Corresponding author

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SH2 domain proteins are important components of the signal transduction pathways activated by growth factor receptor tyrosine kinases. We have been cloning SH2 domain proteins by bacterial expression cloning using the tyrosine phosphorylated C-terminus of the epidermal growth factor receptor as a probe. One of these newly cloned SH2 domain proteins, GRB-7, was mapped on mouse chromosome 11 to a region which also contains the tyrosine kinase receptor, HER2/erbB-2. The analogous chromosomal locus in man is often amplified in human breast cancer leading to overexpression of HER2. We find that GRB-7 is amplified in concert with HER2 in several breast cancer cell lines and that GRB-7 is overexpressed in both cell lines and breast tumors. GRB-7, through its SH2 domain, binds tightly to HER2 such that a large fraction of the tyrosine phosphorylated HER2 in SKBR-3 cells is bound to GRB-7. GRB-7 can also bind tyrosine phosphorylated SHC, albeit at a lower affinity than GRB2 binds SHC. We also find that GRB-7 has a strong similarity over >300 amino acids to a newly identified gene in *Caenorhabditis elegans*. This region of similarity, which lies outside the SH2 domain, also contains a pleckstrin homology domain. The presence of evolutionarily conserved domains indicates that GRB-7 is likely to perform a basic signaling function. The fact that GRB-7 and HER2 are both overexpressed and bound tightly together suggests that this basic signaling pathway is greatly amplified in certain breast cancers.

Key words: *C.elegans*/erbB-2/pleckstrin domain/SH2 domain/tyrosine kinase

Introduction

Many growth factors bind to receptors with intrinsic tyrosine kinase activity (Schlessinger and Ullrich, 1992; Fantl *et al.*, 1993). These receptors are crucial for normal development but can also act as oncogenes leading to cell transformation. The family of epidermal growth factor receptor (EGF-receptor) related tyrosine kinases illustrates the diverse functions of these receptors. One of the EGF-receptor related tyrosine kinases, the *Caenorhabditis elegans* protein Let-23,

is crucial for proper development of the nematode vulva (Aroian *et al.*, 1990). Other members of this receptor family are more recognized for their role in oncogenesis. The avian oncogene, *v-erbB*, represents a truncated homologue of the mammalian EGF-receptor (Downward *et al.*, 1984). Another receptor with close similarity to EGF-receptor is HER2, also known as c-erbB-2 (Coussens *et al.*, 1985). This receptor was also isolated as the rat oncogene *neu*, an oncogene responsible for chemically induced rat glioblastomas (Bargmann *et al.*, 1986). HER2/erbB-2 is known to be amplified and overexpressed in ~25% of human breast cancers (Slamon *et al.*, 1987, 1989). In many, but not all studies, this HER2 overexpression correlates with a poor prognosis (Elledge *et al.*, 1992).

To understand the mechanism by which these receptor tyrosine kinases are responsible for both normal development and transformation, it is important to understand the basic intracellular signaling mechanisms activated by these tyrosine kinases. Recent studies have indicated an important role for proteins with SH2 domains in growth factor receptor signal transduction (Margolis, 1992; Pawson and Schlessinger, 1993). These proteins bind to the tyrosine phosphorylated growth factor receptors due to a direct interaction between the SH2 domain and the phosphotyrosine-containing peptide sequences within the receptor (Moran *et al.*, 1990; Margolis *et al.*, 1990a). The SH2 domain proteins then trigger several different signaling cascades. Some SH2 domain proteins, such as phospholipase C- γ (PLC- γ), have intrinsic enzymatic activity. The binding of PLC- γ to the growth factor receptor tyrosine kinase triggers the phosphorylation of this enzyme leading to a breakdown of polyphosphoinositides (Rhee and Choi, 1992). Other SH2 domain proteins, such as phosphatidylinositol-3 kinase (PI-3 kinase) associated p85, act as adaptors coupling growth factor receptors to other signaling molecules. As an example, p85 serves as a bridge binding growth factor receptors to the 110 kDa PI-3 kinase (Hiles *et al.*, 1992).

Several SH2 domain proteins were isolated in our laboratory by screening bacterial expression libraries with the tyrosine phosphorylated C-terminus of the EGF-receptor (Skolnik *et al.*, 1991; Lowenstein *et al.*, 1992; Margolis *et al.*, 1992). We called this method CORT for cloning of receptor targets. The proteins cloned were called GRBs (growth factor receptor bound). GRB1 was identified as the PI-3 kinase-associated p85 (Skolnik *et al.*, 1991). The second protein isolated using this method was termed GRB2 and consisted of one SH2 domain sandwiched between two SH3 domains (Lowenstein *et al.*, 1992). GRB2 is a homologue of the *C.elegans* protein sem-5 (Clark *et al.*, 1992). Sem-5 is downstream of the receptor tyrosine kinase Let-23 and like Let-23 is crucial for vulval development. Similarly, a *Drosophila melanogaster* homologue of GRB2 and sem-5, Drk, is necessary for proper eye development where it lies downstream of the sevenless tyrosine kinase receptor (Olivier *et al.*, 1993; Simon *et al.*, 1993). Through a combination

Table I. List of loci mapped in interspecific backcross animals

Locus	Probe name	Restriction enzyme	Restriction fragment sizes	
			AEJ/Gn fragment sizes (kb)	<i>M. spretus</i> fragment sizes (kb) ^a
<i>Ngfr</i>	p5b	<i>Pst</i> I	4.0, 2.9, 2.4, 1.5, 1.2	5.5, 2.4, 1.9, 1.5, 1.2
<i>Grb-7</i>		<i>Taq</i> I	2.6	3.8
<i>ErbB-2</i>	neuc(t)/sp6300	<i>Pst</i> I	5.1	4.4
<i>Csf</i> g	pBRG-4	<i>Taq</i> I	3.0	2.5
<i>D11Mit10</i> ^b			0.096	0.126

^aBold restriction fragment size indicates the segregating allele(s) that was typed in the backcross.

^bLocus typed by PCR amplification of microsatellite sequences.

of biochemical and genetic studies it has been determined that GRB2 serves as a crucial link between receptor tyrosine kinases and ras. GRB2 acts as an adaptor forming a complex between activated receptor tyrosine kinases and son of sevenless (SOS), a ras GTP/GDP exchange protein (Buday and Downward, 1993; Chardin *et al.*, 1993; Egan *et al.*, 1993; Gale *et al.*, 1993; Li *et al.*, 1993; Olivier *et al.*, 1993; Simon *et al.*, 1993).

Recently, using CORT, we identified another novel SH2 domain protein called GRB-7 (Margolis *et al.*, 1992). GRB-7 has a single SH2 domain at its C-terminus, a central region with similarity to ras GAP and a proline rich N-terminus. In this paper, we report that GRB-7 maps to a region on mouse chromosome 11 containing the gene for the HER2/erbB-2 receptor tyrosine kinase. This region of mouse chromosome 11 is syntenic to an area of human chromosome 17q that is often amplified in breast cancer (Slamon *et al.*, 1987; van de Vijver *et al.*, 1987; Buchberg *et al.*, 1989). Accordingly, we examined GRB-7 expression in breast cancer. We find that GRB-7 is amplified and overexpressed in breast cancer in concert with the HER2 receptor. GRB-7 is bound tightly to HER2 via its SH2 domain such that the majority of tyrosine phosphorylated HER2 is complexed with GRB-7. Finally, we demonstrate that GRB-7 contains a region of >300 amino acids with similarity to a *C.elegans* gene recently identified by the *C.elegans* genome project (Sulston *et al.*, 1992). This region lies outside the SH2 domain and is likely crucial in GRB-7 signal transduction.

Results

GRB-7 localizes to mouse chromosome 11 near HER2/erbB-2

To determine GRB-7 chromosomal localization, liver genomic DNA from inbred and recombinant inbred (RI) strains of mice was digested with *Taq*I and typed by Southern blotting as described previously (Sap *et al.*, 1990). This analysis revealed two allelic forms of GRB-7, whose inheritance in RI strains of mice defined a single locus. Comparison of the strain distribution pattern for GRB-7 with those for 1124 other markers distributed over all of the mouse chromosomes allowed GRB-7 to be localized to chromosome 11 on the basis of its tight linkage to markers such as *HoxB* (one recombinant among 31 informative RI strains scored), *Gfap* (five recombinants among 52 strains) and *Krt-1* (one recombinant among 31 strains).

To refine the localization of GRB-7, we determined its location on mouse chromosome 11 in an interspecific backcross, that has been typed for >30 genetic markers

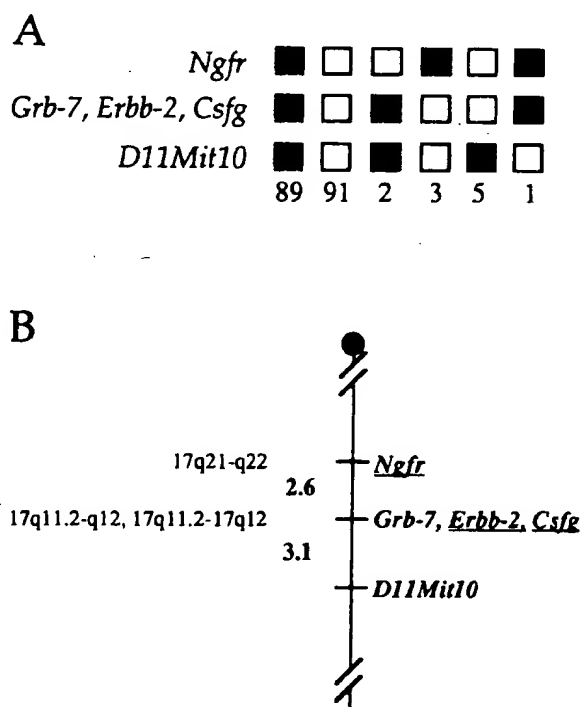


Fig. 1. GRB-7 maps on chromosome 11 near to HER2/erbB-2. (A) Summary of the results of the interspecific backcross analysis. Genes mapped in the analysis are listed on the left. Each column represents the chromosome identified in the N2 progeny inherited from the (AEJ/GN \times *M. spretus*) F1 parent. The closed boxes represent the AEJ/GN allele and the open boxes represent the *M. spretus* allele. The numbers of each type of chromosome identified in the backcross progeny are listed at the bottom. (B) Genetic localization of GRB-7. The figure represents the region of mouse chromosome 11 analyzed in the interspecific backcross. The genes mapped are listed on the right and the genetic map distance (in cM) between adjacent loci, are listed on the left of the chromosome. The location of the human homologues of the genes are listed on the extreme left.

distributed throughout its length. Genomic DNAs from AEJ/Gn and *Mus spretus* parental control animals were digested with several restriction endonucleases and analyzed by Southern blot hybridization using probes that identified the following loci: *Ngfr*, *Grb-7*, *ErbB-2* and *Csf*g (Table I). At least one restriction fragment length polymorphism (RFLP) was identified for each of the probes tested and the sizes of the genomic restriction fragments detected by each probe are listed in Table I. To determine the size polymorphism for *D11Mit10*, parental DNAs and F1 controls

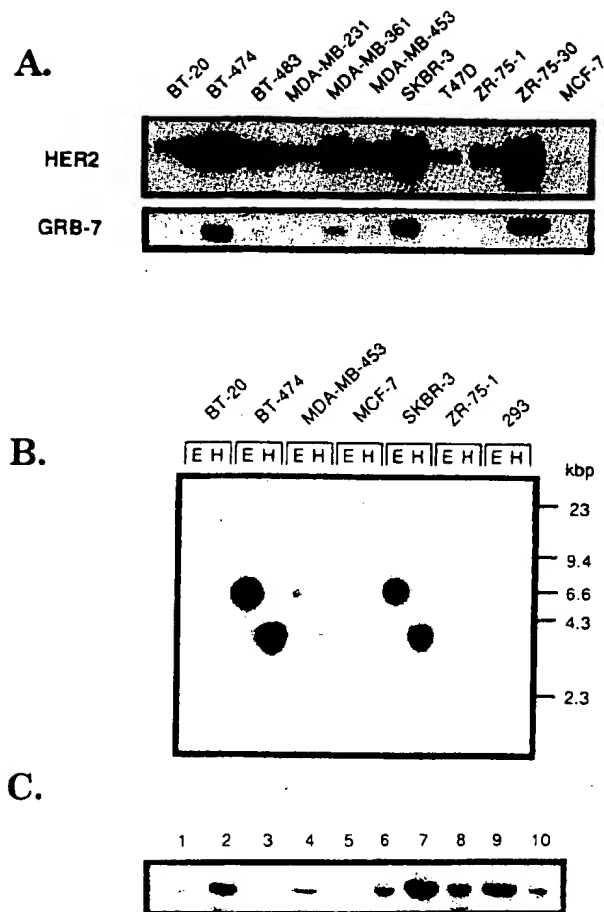


Fig. 2. GRB-7 is amplified and overexpressed in breast cancer cell lines. (A) Immunoblotting of GRB-7 in breast cancer cell lines. Protein (200 μ g) from cell lysates was run on an SDS gel and transferred to nitrocellulose. The nitrocellulose was then blocked and probed with either anti-GRB-7 (#188) antibody or anti-HER2 antibody. After detection with [125 I]protein A, blots were exposed to film. The HER2 blot was exposed for 4 h and the GRB-7 blot for 12 h. (B) Southern blotting of GRB-7 in breast cancer cell lines. Genomic DNA (10 μ g) was digested with either *Eco*RI (E) or *Hind*III (H) and separated on a 0.7% agarose gel. All lanes represent breast cancer cell lines except 293 cells (human embryonic kidney), which were used as a control. Blotting and hybridization were carried out with a GRB-7 probe as described in Materials and methods. (C) Immunoblotting of GRB-7 in breast tumors. Frozen pulverized tumor powder was lysed in 5% SDS as described (Tandon *et al.*, 1989) and 100 μ g of total solubilized protein was run on SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-GRB-7 (#188) antibody. After detection with [125 I]anti-rabbit sera (Amersham) the nitrocellulose blots were exposed to film. A representative blot of 10 tumors is shown. All tumors overexpressed HER2 except #5. With the exception of #3 and #5, all tumors were considered positive for GRB-7 expression.

were polymerase chain reaction (PCR) amplified using the oligomers defining *D11Mit10*. As expected, a simple sequence length polymorphism (SSLP) was detected between AEJ/Gn and *M.spretus* (Table I).

The segregation pattern of the *M.spretus* allele in 191 backcross animals was then determined for *Ngfr*, *Grb-7*, *ErbB-2* and *CsfG* (by Southern blot hybridization) and for *D11Mit10* by PCR amplification and agarose gel electrophoresis. The results are summarized in Figure 1. Mice were either homozygous for the AEJ/Gn allele or heterozygous for the *M.spretus* and AEJ/Gn alleles. The

Table II. Correlation of GRB-7 expression with HER2 overexpression in human breast tumors^a

	HER2 negative ^b	HER2 positive
GRB-7 negative	30	10
GRB-7 positive	8	24

^aSignificance $P < 0.001$

^bHER2 overexpression was determined by immunoblotting as previously described (Ciocca *et al.*, 1992)

frequency of AEJ/Gn and *M.spretus* alleles for the loci mapped in the N2 progeny did not significantly differ from the expected 1:1 ratio. The results presented here are consistent with the previous genetic localization of *Ngfr*, *ErbB-2* and *CsfG* using a [(C57BL/6J \times *M.spretus*) \times C57BL/6J interspecific backcross (Buchberg *et al.*, 1989). Gene order was determined by minimizing the number of multiple recombinants between loci. The order of the loci and the ratio of the number of recombinants to the total number of N2 offspring examined for each locus are: *Ngfr* - 5/191 - (*Grb-7*, *ErbB-2*, *CsfG*) - 6/191 - *D11Mit10*. The genetic distances between the loci in centimorgans \pm standard error are: *Ngfr* - 2.6 ± 1.1 - (*Grb-7*, *ErbB-2*, *CsfG*) - 3.1 ± 1.2 - *D11Mit10*. No recombinants were detected between *Grb-7*, *ErbB-2* and *CsfG* in 191 N2 progeny, indicating that these loci are tightly linked and must lie < 1.6 cM apart (upper 95% confidence limit).

GRB-7 is amplified and overexpressed in breast cancer cell lines

Next, we determined if GRB-7 is amplified and overexpressed with HER2 in breast cancer. We checked the expression of GRB-7 in several breast cancer cell lines by immunoblotting cell lysates for GRB-7 expression. We found a close correlation between HER2 and GRB-7 expression (Figure 2A). To confirm that this overexpression was due to gene amplification, we performed Southern blotting with a GRB-7 probe (Figure 2B). GRB-7 is amplified in both SKBR-3 cells and BT-474 - two cell lines in which HER2 is also amplified (Kury *et al.*, 1990). Neither GRB-7 nor HER2 is amplified in the other cell lines.

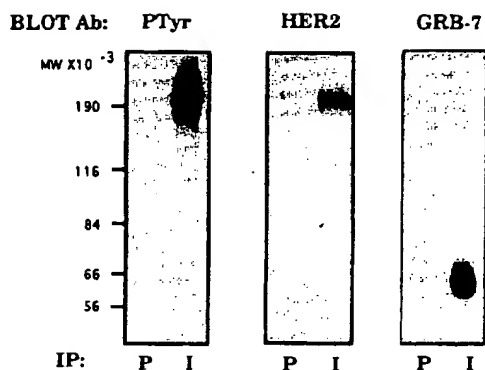
GRB-7 is overexpressed in breast cancer tissue

Immunoblotting with the GRB-7 antibody was then performed on 72 breast cancer samples in which HER2 expression had previously been analyzed, also by an immunoblot procedure (Ciocca *et al.*, 1992). A representative result for 10 such tumors is displayed in Figure 2C. Thirty-four of the breast cancer specimens exhibited HER2 overexpression, and 24 of these were concomitantly positive for GRB-7 expression (Table II). In the 38 tumors negative for HER2 overexpression, only eight were positive for GRB-7. These results were statistically significant by chi-square analysis ($P < 0.001$), and they confirm the cell line data, suggesting that GRB-7 is often expressed in human breast tumors in concert with HER2 overexpression.

GRB-7 binds tightly to HER2

We next examined whether the GRB-7 protein was physically associated with the HER2 receptor. This might be expected as GRB-7 was cloned based on its ability to bind to the EGF-receptor, a receptor closely related to HER2 (Coussens *et*

A.



B.

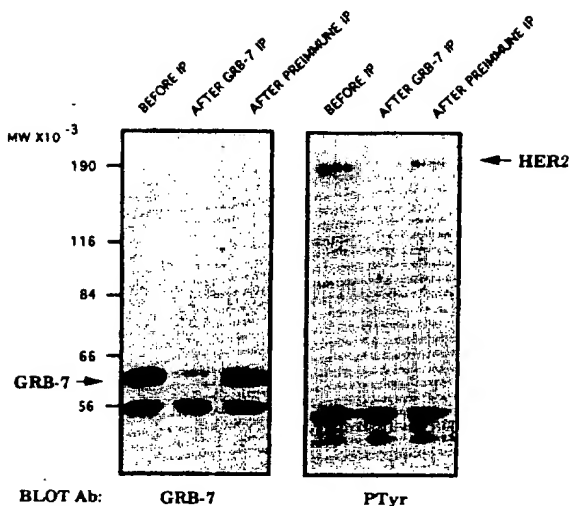


Fig. 3. GRB-7 is tightly bound to HER2. (A) Coimmunoprecipitation of HER2 and GRB-7. SKBR-3 cells were starved overnight in serum free medium and then lysed in 1% Triton X-100 lysis buffer with phosphatase and protease inhibitors. Lysates were then immunoprecipitated with anti-GRB-7 (I) or pre-immune (P) serum and separated by SDS-PAGE. After transfer to nitrocellulose, blots were probed with phosphotyrosine (PTyr), HER2 or GRB-7 (#188) antibodies. Blots were detected as in Figure 2. HER2 and PTyr blots represent immunoprecipitations from 2.5 mg of cellular protein while the GRB-7 blot is an immunoprecipitation from 1 mg of protein. GRB-7 and PTyr blots were exposed for 14 h while the HER2 blot was exposed for 4 h. (B) GRB-7 immunoprecipitation clears tyrosine phosphorylated HER2 from SKBR-3 cell lysates. Cell lysate (500 mg) from starved SKBR-3 cells was immunoprecipitated with 20 μ l of pre-immune or anti-GRB-7 (#222) serum. Lysates (130 μ g) from before and after the immunoprecipitations were then immunoblotted with anti-PTyr and anti-GRB-7 (#188) to determine what fraction of GRB-7 and tyrosine phosphorylated HER2 was cleared from the lysate by immunoprecipitation. The band at 56 kDa in the GRB-7 blot is an unidentified protein recognized by affinity purified antibody #188. It is not recognized by any of the other GRB-7 antibodies in immunoprecipitation or immunoblotting. Exposure time was 14 h.

et al., 1985). To perform these studies, GRB-7 was immunoprecipitated from serum-starved SKBR-3 cells and then blotted with phosphotyrosine or HER2 antibodies. Antibodies to GRB-7 specifically immunoprecipitated a phosphotyrosine-

containing band at 190 kDa (Figure 3A, left panel) which was identified as HER2 (Figure 3A, middle panel). This co-immunoprecipitation was not seen with pre-immune serum. No other bands were detected in the antiphosphotyrosine blot, indicating that GRB-7 was not tyrosine phosphorylated (Figure 3A, middle and right panels). This co-immunoprecipitation between GRB-7 and HER2 was demonstrated with three different GRB-7 antisera, indicating it was not dependent on the antibody used. The co-immunoprecipitation of GRB-7 and HER2 was also seen in BT474 cells (results not shown).

To determine what percentage of HER2 was bound to GRB-7 in these cells, we immunoprecipitated GRB-7 from a small number of cells such that we depleted the lysate of GRB-7. Under these conditions, the lysates were almost completely cleared of tyrosine phosphorylated HER2 as well as GRB-7 (Figure 3B). Pre-immune serum which did not immunoprecipitate GRB-7 did not significantly affect the tyrosine phosphorylated HER2 in the lysates. It should be noted that GRB-7 immunoprecipitation did not measurably affect the total amount of HER2 in the cell lysate as only a small fraction of HER2 is tyrosine phosphorylated and able to bind GRB-7. These results indicate a strong association between tyrosine phosphorylated HER2 and GRB-7.

GRB-7 binds SHC

We next asked whether GRB-7 became tyrosine phosphorylated after ligand stimulation of growth factor receptors. Because the true ligand or ligands for HER2 is still unclear (Peles *et al.*, 1993; Plowman *et al.*, 1993), we stimulated tyrosine phosphorylation by activation of the EGF-receptor in SKBR-3 cells (King *et al.*, 1988). After stimulation with EGF, several additional bands become tyrosine phosphorylated yet tyrosine phosphorylation of GRB-7 was still undetectable (Figure 4). Phosphoamino acid analysis of GRB-7 before and after stimulation revealed the presence of phosphoserine and phosphothreonine but no phosphotyrosine (results not shown).

We did find that GRB-7 antibodies immunoprecipitated a tyrosine phosphorylated protein of 54 kDa after EGF stimulation (Figure 4). We suspected that this 54 kDa band might be tyrosine phosphorylated SHC (Pellicci *et al.*, 1992) as it has been found that another SH2 domain protein, GRB2, binds tightly to phosphorylated SHC (Rozakis-Adcock *et al.*, 1992; Skolnik *et al.*, 1993). To confirm this suspicion, we immunoprecipitated GRB-7 from EGF-stimulated SKBR-3 cells and immunoblotted with anti-SHC antibodies (Figure 4, right panel). We were able to detect EGF-stimulated association of SHC and GRB-7.

We next wanted to determine whether GRB-7 binds directly to HER2 and SHC through the GRB-7 SH2 domain. The association of the GRB2 SH2 domain with SHC presumably occurs due to the tyrosine phosphorylation of SHC at residue 317 (Rozakis-Adcock *et al.*, 1992; Skolnik *et al.*, 1993; Songyang *et al.*, 1993). The sequence around this tyrosine, YVN, is felt to represent a high affinity binding site for the SH2 domain of GRB2 when tyrosine phosphorylated. A similar motif is present in EGF-receptor at Tyr1068 (YIN) and in HER2 at Tyr1139 (YVN). We suspected that the GRB-7 SH2 domain might also bind to both HER2 and SHC via this motif. To study this problem, we prepared a GST fusion protein of the GRB-7 SH2 domain containing a protein kinase A phosphorylation site (Ron and

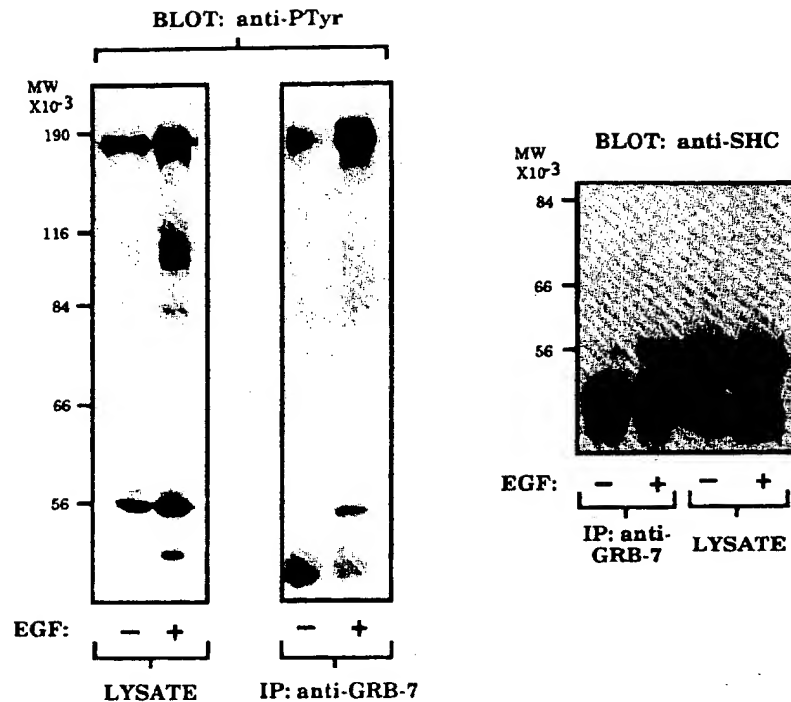


Fig. 4. GRB-7 antisera co-immunoprecipitates SHC from EGF-stimulated SKBR-3 cell lysates. SKBR-3 cells were starved in serum free media overnight and then treated with or without 200 nM EGF for 3 min. After cell lysis, immunoprecipitation was performed with anti-GRB-7 (# 222) or anti-SHC antibodies on 1.5 mg of cell lysate. Immunoprecipitates and cell lysate (130 μ g) were then run on SDS-PAGE and transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine (PTyr), or anti-SHC antibodies. In the SHC immunoblot, the band at ~50 kDa represents the IgG heavy chain in the GRB-7 immunoprecipitate while in the cell lysate it represents the 46 kDa form of SHC.

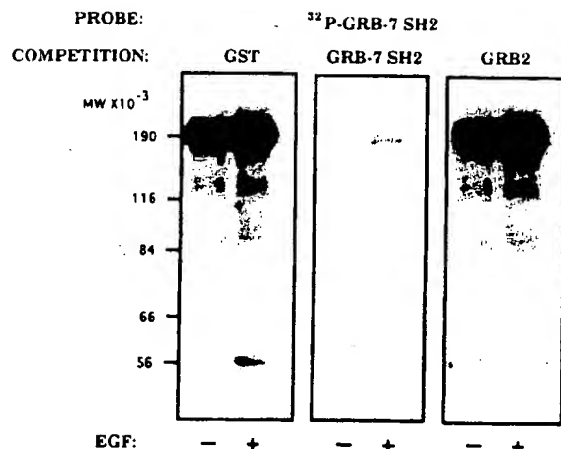
Dressler, 1992). Phosphorylation by protein kinase A in the presence of [γ -³²P]ATP was then used to label the GST fusion protein. SKBR-3 lysates were separated by SDS-PAGE, transferred to nitrocellulose and probed with the labeled GRB-7 SH2 domain. During these incubations, we added a 100-fold molar excess of unlabeled GST, GST-GRB-7-SH2 or GST-GRB2. With GST alone, the GRB-7 SH2 domain bound to two major bands at 190 kDa and 54 kDa, corresponding to HER2 and SHC (Figure 5A, left panel). This demonstrates that GRB-7 can bind directly to these proteins in cells and does not require intermediate molecules. As expected, unlabeled GRB-7 competed the binding to both proteins (Figure 5A, middle panel). We found that GRB2 could prevent the GRB-7 SH2 domain from binding to SHC but did not affect HER2 binding (Figure 5A, right panel).

When we probed the same lysates with labeled GRB2 at a similar concentration and specific activity as the GRB-7-SH2 domain, GRB2 showed strong binding to SHC but no binding to HER2 (Figure 5B). We did detect binding of GRB2 to a protein at 160 kDa which is not HER2 but likely represents the association of the GRB2 SH3 domains to SOS (Buday and Downward, 1993; Egan *et al.*, 1993; Li *et al.*, 1993; Rozakis-Adcock *et al.*, 1993). Unlabeled GRB-7 could not compete the binding of GRB2 to SHC in contrast to the fact that GRB2 could easily compete GRB-7 binding to SHC. Taken together, these results indicate that GRB-7 binds SHC probably at the same Y(V/I)N motif as GRB2. However, the affinity of GRB2 for SHC appears much greater than that of GRB-7. In contrast, GRB-7 binds tightly to HER2, but GRB2 does not compete this binding.

GRB-7 is phosphorylated by the EGFR-HER2 chimera
To determine if GRB-7 was tyrosine phosphorylated by activated HER2, we transfected GRB-7 into NIH 3T3 cells containing the chimeric EGFR-HER2 receptor (Lee *et al.*, 1989). These cells contain the HER2 intracellular domain fused to the EGF-receptor extracellular domain such that EGF can stimulate the HER2 tyrosine kinase. After EGF stimulation of these cells, GRB-7 becomes tyrosine phosphorylated and associates with the chimeric receptor in a ligand dependent fashion (Figure 6A). EGF also induces association between GRB-7 and an unidentified tyrosine phosphorylated protein of 70 kDa. We examined the binding of the [³²P]GRB-7 SH2 domain to lysates from NIH 3T3 cells expressing the EGFR-HER2 chimera (Figure 6B). We detected binding to three bands: the chimeric receptor at 190 kDa, the 54 kDa SHC band and the unidentified band at 70 kDa. The binding of the GRB-7 SH2 domain to these proteins was enhanced in lysates from EGF-stimulated cells. There was some binding of the probe to the receptor even in the absence of ligand because the receptor is autophosphorylated to some extent in the untreated cells. GRB2 was able to completely compete the binding of the GRB-7 SH2 domain to SHC but did not significantly affect the binding to the 70 kDa protein. GRB2 weakly competed with GRB-7 for binding to the chimeric receptor.

Primary sequence of GRB-7 reveals homology to a putative gene in *C.elegans* and the pleckstrin domain
Our data indicate that GRB-7 is a signaling partner for HER2 especially in breast cancer cells where both are overexpressed. However, it is not clear what signal might

A.



B.

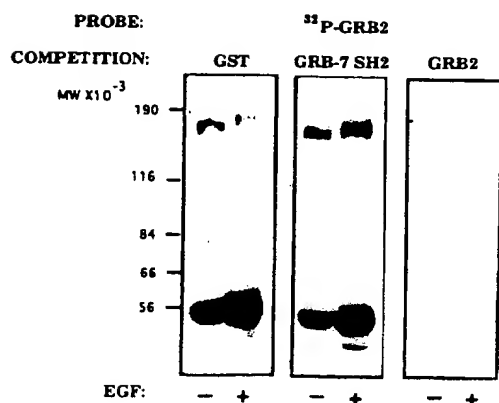


Fig. 5. Binding of GRB-7 SH2 domain and GRB2 to SKBR-3 lysates. GST fusion proteins of GRB-7 SH2 domain and full-length GRB2 were prepared in a GST vector that incorporates a protein kinase A phosphorylation site. The fusion proteins were labeled using protein kinase A and [γ - 32 P]ATP as described in Materials and methods. SKBR-3 lysates, treated with or without EGF, were then separated by SDS-PAGE and transferred to nitrocellulose. These blots were blocked for 2 h at room temperature in 5% non-fat milk and incubated with 12.5 ng/ml (specific activity 2×10^7 c.p.m./ μ g) of probe. Included with the probe was a 100-fold molar excess of either GST, GRB-7 SH2 or GRB2 protein. (A) GRB-7 SH2 domain probe, exposure time 12 h. (B) GRB2 probe, exposure time 2 h.

be sent through GRB-7 when it binds and perhaps becomes phosphorylated by HER2. We have recently detected a close similarity between GRB-7 and a putative gene identified by the *C.elegans* genome sequencing project (Sulston *et al.*, 1992). This putative *C.elegans* gene, F10E9.6, encodes a predicted protein of 650 amino acids with no SH2 domain (Figure 7A). The region of similarity spans ~330 amino acids with an identity of 28% and similarity of 38% (Figure 7B). Using the randomization utility of the alignment program, Bestfit (Devereux *et al.*, 1984), we found that the optimal alignment score for GRB-7 to F10E9.6 lies >18 standard deviations from the mean of scores from 10

scrambled alignments. This indicates a highly significant relationship between the two proteins.

This region of similarity between F10E9.6 and GRB-7 also contains a pleckstrin domain. First described by Mayer and coworkers (Mayer *et al.*, 1993) as well as Haslam and coworkers (Haslam *et al.*, 1993), the pleckstrin domain is found in several different signaling molecules including pleckstrin (Tyers *et al.*, 1988), the serine kinase akt/rac (Bellacosa *et al.*, 1991; Jones *et al.*, 1991) ras GAP (Trahey *et al.*, 1988; Vogel *et al.*, 1988) and the SH3 binding protein, 3BP-2 (Ren *et al.*, 1993). In fact, the similarity we initially detected between ras GAP and GRB-7 encompasses the pleckstrin domain (Margolis *et al.*, 1992). It has been suggested that the pleckstrin motif may function as a protein binding domain (Haslam *et al.*, 1993; Mayer *et al.*, 1993; Musacchio *et al.*, 1993). An alignment of GRB-7, F10E9.6 and the derived consensus sequences for the pleckstrin domains is shown in Figure 7C. A more detailed alignment of these proteins in the context of other pleckstrin domain proteins has also recently been published (Musacchio *et al.*, 1993). While it is not known what signal is relayed by GRB-7, it is clearly a protein composed of evolutionarily conserved domains, indicating that it likely performs a basic signaling function.

Discussion

In this paper we report an amplification and overexpression of GRB-7 in several different breast cancer cell lines. GRB-7 overexpression is also found in tissue samples from primary human breast cancer. This overexpression correlates with HER2 expression indicating that these two genes are often co-amplified and/or co-expressed. Other genes on human chromosome 17q have also been found to be co-amplified with HER2. Although c-erbA-thyroid hormone receptor is co-amplified with HER2, overexpression of this protein has not been detected (van de Vijver *et al.*, 1987; Tavassoli *et al.*, 1989). Topoisomerase IIa has been found to be co-amplified with HER2 but only in a relatively small percentage of cases (Smith *et al.*, 1993). Our results indicate that GRB-7 is likely present in an amplicon which also contains HER2 and which represents a region on 17q. This region on 17q lies proximal to the locus containing the breast cancer susceptibility gene, BRCA1 (Bowcock *et al.*, 1993).

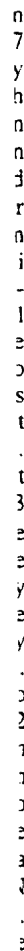
Not only are GRB-7 and HER2 co-expressed in breast cancer but they also exist in a tight complex. In SKBR-3 cells, we co-immunoprecipitated a large fraction of the tyrosine phosphorylated HER2 with GRB-7 antibodies. The fraction of total HER2 that is bound to GRB-7 is relatively low, as only a small percentage of HER2 is tyrosine phosphorylated in these unstimulated cells. GRB-7 is not only bound to HER2 in SKBR-3 cells but can also bind SHC. The binding of GRB-7 to SHC gives us some insight into the tyrosine phosphorylation sites to which the GRB-7 SH2 domain can bind. It seems likely that the GRB-7 SH2 domain binds the Y(V/I)N motif on SHC with a lower affinity than GRB2. GRB2, which competes with GRB-7 for binding to SHC, does not affect the binding of GRB-7 to HER2 in the SKBR-3 cells. This suggests that GRB-7 either binds to a site other than Y(V/I)N on HER2 or binds to the Y(V/I)N site on HER2 but with a much greater affinity than GRB2. It should be noted that GRB2 does not bind HER2 from the

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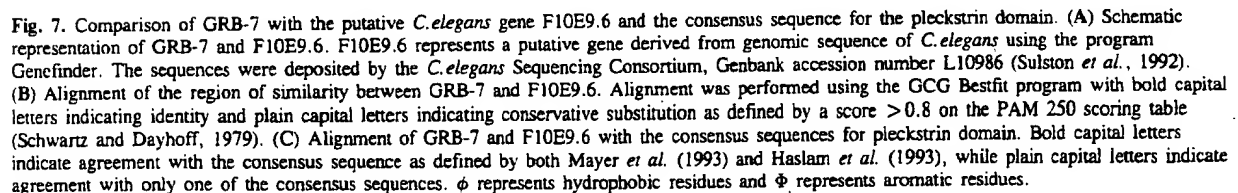
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SKBR-3 cells yet binds to the EGFR-HER2 chimeric receptor after EGF stimulation (J.Wu and B.Margolis, unpublished results). This suggests that the intensity or sites of autophosphorylation on the EGFR-HER2 chimera may be different than on HER2 from SKBR-3 cells.

The tyrosine phosphorylation sites of HER2 and EGF-receptor are closely related in sequence (Hazan et al., 1990; Segatto et al., 1990). Thus it is likely that GRB-7 binds to the same site on HER2 and EGFR. However, attempts to inhibit the binding of GRB-7 to HER2 using tyrosine phosphorylated peptides from the EGF-receptor have been to date, unsuccessful. It has been notoriously difficult to map SH2 domain binding sites on the EGF-receptor in the past, due to the close proximity and overlapping binding of several of the autophosphorylation sites. For example, PLC- γ binds with high affinity to three different autophosphorylation sites (Rotin et al., 1992). Thus further work will be necessary to determine the actual binding preference of the GRB-7 SH2 domain. Other clues to the exact binding site of GRB-7 may come from the identity of the 70 kDa protein which binds to the SH2 domain of GRB-7 in the EGF-stimulated NIH 3T3 cells expressing the EGFR-HER2 chimera. Our data suggest that this protein is not the 66 kDa form of SHC because GRB2 cannot compete this binding. Additionally, we could not detect the 66 kDa form of SHC in GRB-7 immunoprecipitates (J.Wu and B.Margolis, unpublished observations). Other potential candidates for this protein are PTP-1D/Syp (Feng et al., 1993; Vogel et al., 1993) or paxillin (Birge et al., 1993).

In contrast to the results obtained in the SKBR-3 cells, GRB-7 was tyrosine phosphorylated in EGF-stimulated cells expressing chimeric EGFR-HER2 receptor. The phosphorylation state of GRB-7 in breast tumors *in vivo* remains to be determined. Nonetheless, there is a distinct possibility that the binding of GRB-7 to the receptor may be sufficient to initiate the GRB-7 signaling pathway. Several proteins such as GRB2 and PI-3 kinase-associated p85 appear to transduce their signal without tyrosine phosphorylation (Backer et al., 1992; Lowenstein et al., 1992; Carpenter et al., 1993). For these adaptor proteins, the binding of the SH2 domain to the receptor appears sufficient to initiate the signaling cascade.

One of the major questions that remains is the signal relayed by GRB-7. The SH2 domain proteins are grouped into two major classes: those that have intrinsic catalytic or signaling activity and those that act as adaptors coupling secondary proteins to growth factor receptor tyrosine kinases (Margolis, 1992; Pawson and Schlessinger, 1993). The one clue that GRB-7 may act as an adaptor molecule is the fact that GRB-7 contains a sequence similar to the recently described pleckstrin domain. This motif is found in several proteins that already have catalytic activity (Haslam et al., 1993; Mayer et al., 1993; Musacchio et al., 1993). In these proteins, it is theorized that the pleckstrin domain has a role in binding other regulatory factors. One possible example of this function is the association of the pleckstrin domain on the β -adrenergic receptor kinase with $\beta\gamma$ subunits of heterotrimeric G-proteins (Koch et al., 1993; Musacchio et al., 1993). The amino acid sequence identity between different pleckstrin domains is <20%, so more studies will be required before a uniform function can be assigned to this domain.

The alignment between the putative *C.elegans* gene,

F10E9.6, and GRB-7 is more significant and the two genes likely function in a similar fashion. The F10E9.6 gene is theorized to exist based on the analysis of the *C.elegans* genome sequence using the program Genefinder (Sulston et al., 1992). This similarity includes the pleckstrin domain and a second region which extends toward the N-terminus. It is also interesting to note that GRB-7 and F10E9.6 both have proline rich domains but that F10E9.6 does not appear to have a SH2 domain. Such proline rich regions are potential binding sites for SH3 domains (Li et al., 1993; Ren et al., 1993). One gene that has been mapped to the same region of the *C.elegans* genome as F10E9.6 is *mig-10*. *mig-10* was isolated as a gene involved in longitudinal neuronal migration in the nematode (Manser and Wood, 1990). Certain cells in *C.elegans* such as the canal-associated neuron (CAN) and the hermaphrodite specific neuron (HSN) must undergo long range migrations which they do not complete in the *mig-10* mutants. There are >20 genes known to be required for this process but the identity of these genes is not yet known (Wadsworth and Hedgecock, 1992). There is preliminary evidence that *mig-10* could be encoded by F10E9.6 based on cosmid rescue data and partial cDNA sequence (J.Manser, personal communication). However, further studies on the relationship of F10E9.6, *mig-10* and GRB-7 will be required.

Our data seem to suggest a basic signaling function for GRB-7, yet GRB-7 is expressed only in kidney, liver and gonads, suggesting it may have a more specialized function. Recently, in a CORT screen of NIH 3T3 cells using the ajnik tyrosine phosphorylated EGF-receptor, we have isolated a second gene with high similarity to GRB-7 (J.Ooi, V.Yajnik, D.Immanuel and B.Margolis, unpublished data). This gene, which we call GRB-10, is ~60% identical to GRB-7 through the SH2 domain and the central F10E9.6 domain. GRB-10 appears to be more widely expressed than GRB-7, indicating that the basic signaling function of GRB-7 may be performed by a family of related genes in different tissues.

One point of future interest is the effect of GRB-7 overexpression on breast cancer biology. Currently, our results indicate that GRB-7 expression alone cannot transform NIH-3T3 cells (J.Wu and B.Margolis, unpublished results). In the NIH 3T3 cells which express GRB-7 and the chimeric EGFR-HER2 receptor, our data are inconclusive because these cell lines are already transformed due to the high expression of the chimeric receptor. Whether GRB-7 expression, like HER-2, has prognostic significance in patients with primary breast cancer remains to be seen. Although our data indicate a highly significant correlation between overexpression of HER-2 and overexpression of GRB-7 in patient samples, the relationship is imperfect; 24 of the 34 specimens overexpressing HER-2 also overexpress GRB-7 but 10 do not. Similarly, about one-third of HER-2 negative tumors still overexpress GRB-7. It is interesting to speculate that those tumors overexpressing both proteins would have particularly aggressive course and worse patient outcome, a hypothesis we are now testing. Aside from their role in cancer, GRB-7 and related genes are likely to have a basic function in signal transduction. A combination of biochemical studies in mammalian cells and genetic studies in *D.melanogaster* and *C.elegans* led to the elucidation of the function of GRB-2. Likewise, a similar combination of genetics and biochemical studies will lead to a better understanding of the function of GRB-7.

Materials and methods

Tissue culture

Breast cancer cell lines were originally obtained from the ATCC and grown in DMEM, 4500 mg/dl glucose with penicillin/streptomycin and 10% fetal calf serum. All NIH 3T3 cells were grown in the same medium but using 10% calf serum. NIH 3T3 cells expressing the EGFR-HER2 chimera were obtained from Drs A. Zilberstein and A. Ullrich. For experiments cells were starved as described in the figure legends.

Immunoprecipitation and immunoblotting

Several rabbit polyclonal antibodies were generated against GRB-7. Three antibodies were generated against GST fusion proteins of GRB-7 consisting of amino acids 419–535 (#191), amino acids 297–535 (#193) and the full-length protein (#222). An antipeptide antibody was generated against amino acid sequence 264–279 (#188). Antibodies were either used as whole serum or were affinity purified. For affinity purification, the fusion protein or peptide was immobilized on Affi-gel 10 (Bio-Rad). Serum was concentrated by 50% ammonium sulfate precipitation and dissolved in 10 mM Tris, pH 7.5. The ammonium sulfate precipitated antibodies were then purified on the antigen column as described (Harlow and Lane, 1988), eluting the antibody with 100 mM glycine, pH 2.5. Immunoprecipitation and immunoblotting in the cell lines were performed as previously described (Margolis *et al.*, 1989). Immunoblotting of the breast tumor samples with the GRB-7 antibody was performed using 100 µg of sodium dodecyl sulfate-solubilized protein extract as previously described (Tandon *et al.*, 1989). SHC antibodies were purchased from Signal Transduction Labs (Lexington, Kentucky). Rabbit polyclonal antibodies directed against the C-terminus of HER2 were generously provided by Dr A. Zilberstein. Rabbit polyclonal antiphosphotyrosine antibodies were prepared using standard techniques (Kamps and Sefton, 1988).

GRB-7 constructs

GST fusion proteins were generated by PCR of GRB-7 incorporating *Bam*HI restriction sites into the priming oligonucleotides. For the GST-GRB-7 SH2 domain probe, the sequence encoding amino acids 419–535 was amplified, digested with *Bam*HI and ligated into *Bam*HI digested pGSTag vector (Ron and Dressler, 1992). The pGSTag vector was a gift from D. Ron and the GRB2 pGSTag construct was a gift from E. Lowenstein and J. Schlessinger. For mammalian expression of GRB-7, GRB-7 was cut from the λ EX10x plasmid with *Xba*I and *Mse*I and blunt ended using Klenow fragment. This blunt end fragment was ligated into *Eco*RV digested PMJ30 vector (Margolis *et al.*, 1990b). NIH 3T3 cells were transfected as previously described (Margolis *et al.*, 1990b) using GRB-7 in the antisense direction as a control. The hygromycin-B phosphotransferase gene was cotransfected with the GRB-7 constructs as a selectable marker.

GST blotting

The pGSTag constructs were labeled with [γ -³²P]ATP as described using 0.2 U/µl protein kinase A (Ron and Dressler, 1992). Blots were incubated for 2 h at room temperature in block buffer (20 mM HEPES, pH 7.5, 5 mM MgCl₂, 1 mM KCl, 5 mM DTT, 5% non-fat dry milk and 0.02% sodium azide) and probed for 2 h at room temperature in the same buffer using 12.5 ng (3 × 10⁵ d.p.m.) per ml of probe. Blots were washed four times for 15 min with Tris buffered saline (10 mM Tris pH 7.5, 150 mM NaCl) containing 0.1% Triton X-100 before exposing.

Southern blotting

Genomic DNA was prepared from tissue culture cells using standard techniques (Sambrook *et al.*, 1989). DNA (10 µg) was digested with *Eco*RI or *Hind*III and separated on 0.7% agarose gel. The DNA was transferred to Nytran (Schleicher and Schuell) using capillary action and cross-linked by UV light. The blot was blocked for 4 h at 42°C in hybridization buffer (40% formamide, 10 × Denhardt's, 1% SDS, 6 × SSC and 100 µg/ml salmon sperm DNA) and then incubated with probe (2 × 10⁶ d.p.m./ml) overnight at 42°C. The probe consisted of a PCR product from mouse GRB-7 encompassing nucleotides 1437–1909. The probe was labeled with [³²P]dCTP using a random priming kit (USB). The blots final wash was 0.5 × SSC, 0.1% SDS at 42°C.

DNA analysis

All DNA and protein database searches were performed using the University of Wisconsin Genetics Computer Group Sequence Analysis Software (GCG) package (Devereux *et al.*, 1984). The Genbank and EMBL databases were searched using Fasta and Tfasta, respectively (Pearson and Lipman, 1988). Protein alignments were performed with the GCG program, Bestfit.

Conservative substitutions were defined as a score of ≥ 0.8 using the scoring table of Schwartz and Dayhoff (1979), as modified by Gribskov and Burgess (Gribskov *et al.*, 1984).

Chromosome mapping

The interspecific backcross between (AEJ/Gn-a bPH/abPH × *M. spretus*) F1 × AEJ/Gn-a bPH/abPH was previously described (Marini *et al.*, 1993). Genomic DNA extractions, restriction endonuclease digestions, agarose gel electrophoresis, Southern blot transfers, hybridizations and washes were as described (Ma *et al.*, 1993). DNA oligonucleotides used for detecting the SSLP marker were made using an Applied Biosystems Model 393 DNA synthesizer. SSLP markers were detected by amplifying genomic DNA from N2 animals using the specified DNA oligonucleotide pairs (Dietrich *et al.*, 1992) and Taq DNA polymerase as described (Ma *et al.*, 1993). The results of the interspecific backcrosses were analyzed by calculating the maximum likelihood estimates of linkage parameters as described (Green, 1981).

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